Prolipoprotein Modification and Processing Enzymes in Escherichia coli*

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Prolipoprotein signal peptidase, a unique endopeptidase which recognizes glycyl glyceride cysteine as a cleavage site, was characterized in an in vitro assay system using purified prolipoprotein as the substrate. This enzyme did not require phospholipids for its catalytic activity and was found to be localized in the inner cytoplasmic membrane of the Escherichia coli cell envelope. Globomycin inhibited this enzyme activity in vitro with a half-maximal inhibiting concentration of 0.76 nM. Nonionic detergent, such as NIKkol or Triton X-100, was required for the in vitro activity. The optimum pH and reaction temperature of prolipoprotein signal peptidase were pH 7.9 and 37–45 °C, respectively.

Phosphatidylglycerol:proliprotein glyceryl transferase (glyceryl transferase) activity was measured using [2-3H]glycerol-labeled JE5305 cell envelope and [35S]cysteine-labeled MM18 cell envelope as the donor and acceptor of glyceryl moiety, respectively. 3H and 35S dual-labeled glyceryl cysteine was identified in the product of this enzymatic reaction. The optimal pH and reaction temperature for glyceryl transferase were pH 7.8 and 37 °C, respectively.

A Gram-negative bacterium contains four subcellular compartments, the cytoplasm, the inner cytoplasmic membrane, the periplasmic space, and the outer membrane. Proteins localized in the periplasmic space and in the outer membrane must be translocated across the inner membrane before reaching their final destinations. Most of the periplasmic and outer membrane proteins in Escherichia coli are synthesized as precursor proteins which contain 20–30 amino acid extensions, the so-called signal peptides, at their NH2 termini (1–3). Signal peptides are presumably involved in the initial interaction between the precursor proteins and the putative secretory machinery in the inner membrane and are eventually cleaved by the so-called signal (leader) peptidase either co-translationally or post-translationally (4, 5).

There is a unique group of secreted proteins in E. coli which contains covalently linked lipids at their NH2 termini (6). The murein lipoprotein in E. coli is a prototype of membrane lipoproteins in both Gram-negative and Gram-positive bacteria (7, 8). They are first synthesized as precursor forms but require additional modification and processing reactions before they are converted to the mature lipoproteins.

We have succeeded in the demonstration of these overall modification and processing reactions post-translationally in an in vitro assay using unmodified prolipoprotein and phospholipids as the substrates (9). We have further shown by this in vitro system that modification of prolipoprotein takes place prior to the cleavage of signal peptide (9), and the modified cysteine residue probably constitutes part of the recognition site for the unique PLP signal peptidase (10).

Recently, the plasmid pLC3-13 from Carbon-Clark collection was identified to carry PLP signal peptidase gene (lap) by us (11) and by Yamagata et al. (12) independently. The lap gene was subcloned into PB32 to obtain pMT32, and the level of PLP signal peptidase activity in the cells harboring this plasmid was about 20 times higher than that in the wild-type cells (11).

The availability of cloned plasmid will facilitate the identification and characterization of this enzyme. In the meantime, the establishment of optimal assay conditions and characterization of some basic properties of these enzymes involved in the modification and processing of lipoproteins will be required for the elucidation of the precise mechanisms of biosynthesis and assembly of lipoproteins.

In this paper, we describe the optimal assay conditions and some unique enzymatic properties of PLP signal peptidase and glyceryl transferase. PLP signal peptidase activity is localized in the cytoplasmic membrane fraction of E. coli. In contrast to the signal peptidase activities from eukaryotic cells (13, 14), PLP signal peptidase does not require phospholipids for its catalytic activity. The half-maximal inhibitory concentration of globomycin was determined to be 0.76 nM.

EXPERIMENTAL PROCEDURES AND RESULTS

Subcellular Localization of Prolipoprotein Signal Peptidase—The precursor forms of exported proteins in E. coli are recognized and processed by two distinct signal peptidases; modified lipoproteins are processed by PLP signal peptidase (10) and most, if not all, of other precursor proteins are cleaved by PCP signal peptidase (leader peptidase) purified by Zwizinski and Wickner (15). Determinations of the subcellular localizations of these signal peptidases are important to our understanding of the function of the signal peptides in E. coli.

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1 The abbreviations used are: PLP, prolipoprotein; UPLP, unmodified prolipoprotein; MPL, modified prolipoprotein; PCP, procoag protein; KDO, 2-keto-3-deoxyoctonate; SDS, sodium dodecyl sulfate.

2 Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 5–12, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2408, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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the translocation of proteins. The PCP signal peptidase was reported to be localized equally in both inner and outer membranes (16). To determine the subcellular localization of PLP signal peptidase, outer and cytoplasmic membranes were separated in sucrose density gradient. As shown in Fig. 1, PLP signal peptidase was exclusively located in the cytoplasmic membrane and essentially no activity was detected in the outer membrane. This result indicates that the signal peptide in prolipoprotein is removed prior to the translocation of mature lipoprotein from the inner membrane to the outer membrane (17, 18).

**Prolipoprotein Signal Peptidase Does Not Require Phospholipids for Its Activity**—Some membrane-bound enzymes, including eucaryotic signal peptidases from dog pancreas (13) and hen oviduct (14), require phospholipids for their activity. Since PLP signal peptidase activity was more stable against heat treatment in membrane than in the micelles of Nikkol (Fig. 7), the phospholipids requirement of PLP signal peptidase was studied.

JE5505 membrane (200 μg of protein) was mixed with [2-3H]glycerol-labeled phospholipid vesicles and solubilized with 1% Nikkol. Polyacrylamide gel electrophoresis under non-denaturing conditions was carried out (19), and the gel was sliced for the measurements of both PLP signal peptidase activity and [2-3H]glycerol radioactivity. As shown in Fig. 2, 3H-phospholipids remained at the top portion of the gel while PLP signal peptidase activity was detected at a position of $R_p = 0.3-0.5$. This result clearly shows that PLP signal peptidase is active without phospholipids because the reaction mixture does not contain any phospholipids. In this respect, PLP signal peptidase activity differs from the eucaryotic signal peptidases.

**Globomycin Effect on Prolipoprotein Signal Peptidase in Vitro**—The cyclic antibiotic globomycin is a specific inhibitor of PLP signal peptidase (20, 21). Various concentrations of globomycin dissolved in 0.25% Nikkol was added to the reaction mixture of PLP signal peptide assay in vitro. As shown in Fig. 3, 5 x 10^{-4} μg/ml (0.76 nM) of globomycin inhibits 50% of PLP signal peptidase activity under standard assay conditions. The concentrations of MPLP in reaction mixture was calculated to be approximately 330 nM with the following assumptions; generation time of *E. coli* B (Arai) was 120 min, number of free-form lipoprotein molecules per cell is 5 x 10^9, and PLP synthesis is not affected for 10 min after the addition of globomycin. Half-inhibition of PLP signal peptidase activity by globomycin was obtained at 1/400 molar ratio of globomycin/substrate. Furthermore, *E. coli* strains overproducing PLP signal peptidase by globomycin was obtained at 1/400 molar ratio of globomycin/substrate. Furthermore, *E. coli* strains overproducing PLP signal peptidase by means of chromosomal mutation (22) or high copy number plasmids containing PLP signal peptidase (11) overcome the globomycin effect and show globomycin-resistant phenotype. These data suggest that globomycin binds to the enzyme rather than to the substrate (MPLP).

**Kinetics of Modification and Processing of Newly Synthesized...**
sized Prolipoprotein in MM18 Cells—The donor and acceptor in the glyceryl transferase reaction have been shown to be the nonacylated glycerol moiety of phosphatidylglycerol (23) and the free sulfhydryl group of cysteine at the 21st position of prolipoprotein (9). Unmodified prolipoprotein accumulated in E. coli MM18 cells when the synthesis of malE-lacZ hybrid protein was induced with maltose (9). The kinetics of modification and processing of newly synthesized unmodified prolipoprotein was studied in vivo by a pulse-chase experiment (Fig. 4). Approximately 15% of newly synthesized prolipoprotein was modified and processed during the 15-s pulse and 85% of pulse-labeled prolipoprotein present as the unmodified form. No significant post-translational modification and processing was observed during a 5-min chase. These data are interpreted as follows. In the inner membrane of MM18 cells which contained excess of malE-lacZ hybrid protein, only 15% newly synthesized prolipoprotein was accessible to glyceryl transferase and consequently modified and processed. The rest of the newly synthesized prolipoprotein remained unmodified and unprocessed even after a prolonged chase (Fig. 4). The accumulated UPL forms of prolipoprotein in crude membrane fraction (9) due to either loosely binding to inner membrane or forming insoluble aggregates (28).

DISCUSSION

The maturation and proper assembly of lipoprotein into the outer membrane requires successive modification and processing reactions. We have established in vitro assays for these modification and processing enzymes and characterized some of their properties. While the lipoprotein is the most abundant protein in E. coli, the activity of glyceryl transferase detected in the in vitro assay is low. To improve the yield, we need to investigate the reaction mechanism of glyceryl transferase, such as requirements of cofactors, the formation of activated intermediate complexes, substrate specificities of the acceptor and the donor, and the conformation of the protein substrates. A modification assay coupled with in vitro prolipoprotein synthesis may yield better results in terms of the extent of the reaction.

For PLP signal peptidase assay, we have established a very convenient system using radiochemically pure substrate prepared by immunoprecipitation. The purification of substrate provides the possibility to reconstitute the in vitro system using purified components. Furthermore, multiple enzyme assays can be handled routinely so that the screening of mutants lacking or overproducing PLP signal peptidase becomes feasible. In fact, we have found overproducing mutant (22) and PLP signal peptidase overproducing clone from Clark-Carbon collections (11) using this in vitro assay method.

Recently, Yamagata reported a PLP signal peptidase assay system using E. coli cell envelope containing modified prolipoprotein from a mutant with temperature-sensitive PLP signal peptidase (24). While this system reported by Yamagata is useful, it has a disadvantage that the enzyme activity is measured at the nonphysiological temperature (60 °C) in order to minimize the leaky mutant signal peptide activity present in the cell envelope containing the substrate. The assay under the nonphysiological conditions may affect some of the properties of this enzyme. In our system, the activity assayed at 60 °C was only 25% of the maximal enzyme activity at the optimal temperature (Fig. 7). Furthermore, Yamagata reported that 1 μg/ml of globomycin was required for half-maximal inhibition of PLP signal peptidase activity by assaying at 60 °C, a globomycin concentration 1000 times of that measured by our assay at 37 °C (Fig. 3). The interaction of globomycin and enzyme is apparently weakened at higher temperature.

In this paper, we described some basic properties of PLP signal peptidase, and the most interesting problem yet to be resolved is the substrate specificity of this enzyme. The fact that the processing of new membrane lipoproteins is all inhibited by globomycin (8) and some of glyceride-modified precursors of these new lipoproteins accumulate in the mutant cell containing temperature-sensitive signal peptide (25) suggests that PLP signal peptidase can process the precursor forms of most, if not all, these lipoproteins. We recently succeeded in the cloning of PLP signal peptide gene in pBR322-derived plasmid vectors (11). The growth of strain JE5505, lacking murein lipoprotein, is more globomycin resistant than wild type strains (26). We have found that strain JE5505 transformed by plasmids containing PLP signal peptidase gene become extremely globomycin resistant (11). This data suggest that these new lipoproteins may also be killing targets of globomycin and are processed by this enzyme. Further characterization of potential substrates for PLP signal peptidase requires the purification of this enzyme.

PLP signal peptidase was found exclusively in the cytoplasmic membrane. This data suggests that glyceryl transferase is also most likely located in cytoplasmic membrane because modification of prolipoprotein by this enzyme is a prerequisite for PLP signal peptidase activity. The accumulation of unmodified prolipoprotein in MM18 cells after maltose induction suggests that at least the active site of glyceryl transferase not be exposed to the cytoplasmic side of inner membrane.

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REFERENCES

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Experimental procedures

Growth and labeling conditions

Growth of the wild-type strain 23555 (formerly 14840) at 37°C on agar plates containing 5% glucose with or without 0.1% yeast extract resulted in a doubling time of 100 min. Cells were harvested by centrifugation at 20,000 rpm for 15 min and washed with 0.1 M Tris-HCl buffer (pH 7.4) at 4°C before use. Enzymes were assayed in the presence of 10 mm Tris-HCl buffer (pH 7.4) at 25°C.

Assay of proteasome activity

Cell extracts were prepared by homogenization of washed cells in 50 mm Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.1% (w/v) Triton X-100. The homogenate was centrifuged at 100,000 × g for 30 min at 4°C, and the supernatant was used as the source of proteolytic activity. The supernatant was dialyzed against 50 mm Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.1% (w/v) Triton X-100. The dialyzed supernatant was used as the source of renaturation and assay conditions were as described above.

Assay of proteasome activity

Proteasome activity was measured by the method of Kallins et al. [12]. Approximately 100 cells of strain 23555 were assayed in 10 mm Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.1% (w/v) Triton X-100. The assay mixture was incubated at 25°C for 30 min. The reaction was stopped by the addition of 0.15 M NaCl and 0.1% (w/v) Triton X-100. The reaction mixture was then centrifuged at 100,000 × g for 30 min at 4°C, and the supernatant was used as the source of renaturation and assay conditions were as described above.

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**Table 1. Detergent requirements for prolipoprotein signal peptides**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>(A) Stabilization (unit)</th>
<th>(B) Assay (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.08</td>
<td>0.38</td>
</tr>
<tr>
<td>SDS</td>
<td>0.24</td>
<td>0.04</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>0.34</td>
<td>0.08</td>
</tr>
<tr>
<td>Ochol.</td>
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<td>0.29</td>
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<tr>
<td>Triton X-100</td>
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<td>0.97</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>Sarkosyl</td>
<td>0.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

(A) Stabilization: 9 pl of RSA55 membrane (5.0 mg protein/ml) was mixed with 1 pl of 1% detergent and centrifuged to remove insoluble materials. The supernatant solution (1 ml) was used for PLP signal peptide assay at the pressure of 0.15 M (final concentration of the detergent was 0.05).

(B) Assay: RSA55 membrane (5.0 mg protein/ml) was assayed for PLP signal peptide activity at the pressure of 0.15, 0.175 and 0.18 of various detergents.

**Fig. 1** Optimal reaction temperature and heat stability of prolipoprotein signal peptides. For the determination of optimal reaction temperature, prolipoprotein signal peptides (pS of cell envelope proteins) was assayed at various temperatures (○). For the determination of heat stability of prolipoprotein signal peptides (pS of cell envelope proteins) was heated at various temperatures for 30 min, and 1 ml of lipoprotein cell envelope was used for signal peptide assay (●, ●) at 37°C.

**Fig. 2** Linearly of prolipoprotein signal peptide activity against protein amounts. Various amounts of RSA55 cell envelope protein were used for the assay of prolipoprotein signal peptide activity.

**Fig. 3** Neutral pH for glycerol transferase. Neutral transferase activity was measured at neutral pH. Buffers used were (A) 10 mM phosphate buffer (●), (B) 50 mM Tris-HCl buffer (○), and (C) 0.1 M Tris-HCl buffer (●) were used.

**Fig. 4** Neutral pH for glycerol transferase. Neutral transferase activity was measured at neutral pH. Buffers used were (A) 10 mM phosphate buffer (●), (B) 50 mM Tris-HCl buffer (○), and (C) 0.1 M Tris-HCl buffer (●) were used.

**Fig. 5** Measurement of PLP signal peptide activity. PLP signal peptide activity was assayed at various pressures. The pressure was determined by the amount of detergent used. (a) Neutrophil detergent (●, ○). (b) Triton X-100 detergent (●, ○). (c) Tween 80 detergent (●, ○). (d) Sarkosyl detergent (●, ○).

**Fig. 6** Measurement of PLP signal peptide activity. PLP signal peptide activity was assayed at various pressures. The pressure was determined by the amount of detergent used. (a) Neutrophil detergent (●, ○). (b) Triton X-100 detergent (●, ○). (c) Tween 80 detergent (●, ○). (d) Sarkosyl detergent (●, ○).

**Fig. 7** Measurement of PLP signal peptide activity. PLP signal peptide activity was assayed at various pressures. The pressure was determined by the amount of detergent used. (a) Neutrophil detergent (●, ○). (b) Triton X-100 detergent (●, ○). (c) Tween 80 detergent (●, ○). (d) Sarkosyl detergent (●, ○).

**Fig. 8** Optimal reaction temperature for glycerol transferase. Glycerol transferase assay was assayed at various temperatures. The enzyme reaction was carried out at the indicated temperature for 1 hr.

**Fig. 9** Time course of glycerol transferase. Glycerol transferase assay was assayed at indicated periods of incubation time. [3H]-glycerol count incorporated into 1,400 pmol of 1-palmitoyl-sn-glycerol was shown.

**Fig. 10** Optimal reaction temperature for glycerol transferase. Glycerol transferase assay was assayed at various temperatures. The enzyme reaction was carried out at the indicated temperature for 1 hr.

**Fig. 11** Optimal reaction temperature for glycerol transferase. Glycerol transferase assay was assayed at various temperatures. The enzyme reaction was carried out at the indicated temperature for 1 hr.

**Fig. 12** Product identification of glycerol transferase. Sample was prepared as described in the text. Electrophoresis was carried out at 500 V for 2.5 hr at room temperature. Standards for glycerol transferase were prepared from wild-type strain I-180.
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